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High-frequency *Ds* remobilization over multiple generations in barley facilitates gene tagging in large genome cereals

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Abstract Transposable elements have certain advantages over other approaches for identifying and determining gene function in large genome cereals. Different strategies have been used to exploit the maize *Activator/dissociation* (*Ac/Ds*) transposon system for functional genomics in heterologous species. Either large numbers of independent *Ds* insertion lines or transposants (TNPs) are generated and screened phenotypically, or smaller numbers of TNPs are produced, *Ds* locations mapped and remobilized for localized gene targeting. It is imperative to characterize key features of the system in order to utilize the latter strategy, which is more feasible in large genome cereals like barley and wheat. In barley, we generated greater than 100 single-copy *Ds* TNPs and determined remobilization frequencies of primary, secondary, and tertiary TNPs with intact terminal inverted repeats (TIRs); frequencies ranged from 11.8 to 17.1%. In 16% of TNPs that had damaged TIRs no transposition was detected among progeny of crosses using those TNPs as parental lines. In half of the greater than 100 TNP lines, the nature of flanking sequences and status of the 11 bp TIRs and 8-bp direct repeats were determined. BLAST searches using a gene prediction program revealed that 86% of TNP flanking sequences matched either known or putative genes, indicating preferential *Ds* insertion into genic regions, critical in large genome species. Observed remobilization frequencies of primary, secondary, tertiary, and quaternary TNPs, coupled with the tendency for localized *Ds* transposition, validates a saturation mutagenesis approach using *Ds* to tag and characterize genes linked to *Ds* in large genome cereals like barley and wheat.

Keywords *Ac/Ds* · Barley · Remobilization · Transposon tagging

Abbreviations

Ac/Ds	Activator/dissociation
EST	Expressed sequence tag
TIR	Terminal inverted repeat
TNP	Transposon insertion line

Introduction

Characterization of mutant alleles provides valuable insights into the genetics of normal physiological functions. Compared to the study of mutations generated randomly with chemicals or irradiation, for example, technical advances in the field of genomics and the explosion of available gene sequences provide tools that lead to increasingly detailed characterizations of individual genes and their physiological roles. Mutagenesis via T-DNA insertion (Azpiroz-Leehan and Feldmann 1997; Krysan et al. 1999; Jeon et al. 2000), RNA interference (RNAi) (Hannon 2002;

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Waterhouse and Helliwell 2003), and transposon tagging (Walbot 2000; May and Martienssen 2003) more readily enables researchers to study specific genes and their functions.

The transposon-based insertional mutagenesis approach has certain advantages over the other approaches for determining gene function. Transposable elements can be mobilized or immobilized on demand and the approach requires only a few initial transformants to generate large numbers of plants carrying transposed elements [transposants (TNPs)] at different locations. In addition, transposons can have bias for genic regions (Cowperthwaite et al. 2002)—important in large genome species—and this can lead to the identification of regulatory sequences as well as introns.

The early work that enables the use for functional genomics of the maize transposable elements, *Activator/dissociation* (*Ac/Ds*), was first reported by McClintock (1949), when she discovered that a locus of chromosome breakage, *Ds*, could move from one position to another in the maize (*Zea mays* L.) genome in the presence of another factor, *Ac*. The 4.6 kb *Ac* element, encoding the 807-amino acid transposase protein (*Ac*TPase), catalyzes transposition of both *Ac* and *Ds* elements (Kunze and Starlinger 1989). However, *Ds* lacks transposase, while retaining the sequences critical for its transposition—namely 11-bp terminal inverted repeats (TIRs) and subterminal regions of 250–300 nucleotides on both ends (Coupland et al. 1988; Varagona and Wessler 1990).

That the maize *Ac–Ds* system is functional in heterologous species was demonstrated in several pioneering studies (Baker et al. 1986; Yoder et al. 1988; Fedoroff 1989). This demonstration led to the development of one- and two-element transposon tagging resources in dicotyledonous species, such as *Arabidopsis* (Van Sluys et al. 1987; Bancroft and Dean 1993) and tomato (Jones et al. 1994; Cooley et al. 1996), and monocotyledonous species, like rice (*Oryza sativa* L.) (Shimamoto et al. 1993; Nakagawa et al. 2000; Upadhyaya et al. 2002; Kolesnik et al. 2004) and barley (*Hordeum vulgare* L.) (Koprek et al. 2000; Scholz et al. 2001). Two-element systems that introduce *Ds* and *AcTPase* separately have advantages over one-element systems that use the entire *Ac* element because in the former case *AcTPase* segregates from the non-autonomous *Ds* element, stabilizing *Ds* in its new location (Fedoroff 1989; Hehl and Baker 1989).

Introduction of the maize *Ac–Ds* transposable element system as a transposon tagging tool into heterologous species (Parinov et al. 1999; Raina et al. 2002) offers unprecedented opportunities to link genes with function by creating and characterizing mutant alleles. Several developmentally important genes were identified using *Ac/Ds*-mediated insertional mutagenesis (for review, see May and Martienssen 2003). In *Arabidopsis* these include *CURLY LEAF* (Goodrich et al. 1997), *FRUITFULL* (Gu et al. 1998), *MEDEA* (Grossniklaus et al. 1998), and *SPOROCYTELESS/NOZZLE* (Yang et al. 1999). In maize they include *knotted1* (Hake et al. 1989) and *opaque2* (Schmidt et al. 1997) and more recently *BRANCHED FLORETLESS* and *ANTHER INDEHISCENCE1* in rice (Zhu et al. 2003, 2004). Most of these genes were isolated using an undirected tagging approach in which large populations of transposon-containing plants were generated and screened for phenotypic abnormalities. Taking advantage of the tendency of *Ac/Ds* to transpose locally (Smith et al. 1996; Parinov et al. 1999; Upadhyaya et al. 2002), a *Ds* element can be placed near a gene of interest, which can then be tagged by localized mutagenesis. Examples demonstrating the power of this localized tagging approach in maize include cloning of *indeterminate1* (Colasanti et al. 1998) and *tasselseed2* (DeLong et al. 1993).

Barley is a true diploid with high homology to other members of the economically important *Triticeae*, particularly wheat. Additionally extensive genomic resources are available for barley, including large numbers of Expressed Sequence Tags (ESTs), an Affymetrix Barley Genome Array and, most importantly for transposon tagging, extensive mapping resources available in databases developed and maintained by the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) and GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml).

A two-element, *Ac/Ds*-based transposon tagging system was developed in barley (McElroy et al. 1997; Koprek et al. 2000, 2001) and Cooper et al. (2004) reported the map positions of 19 lines containing single *Ds* insertions (TNPs). For these and other TNPs to be maximally useful for saturation mutagenesis and tagging of linked genes by *Ds* remobilization, it is essential to confirm that insertions can be remobilized and to understand the factors that affect *Ac/Ds* function in barley. Earlier studies in rice confirmed that loss of or reduced *Ds* activity occurred during generation advance (Nakagawa et al. 2000; Upadhyaya et al. 2002). Other studies in rice of *Ac/Ds* enhancer traps stressed the need for data on the behavior of *Ds* in advanced generations in order to develop effective tagging strategies (Greco et al. 2001, 2003).

The ability of *Ds* to be remobilized during generation advance can be studied both empirically by direct measurements of *Ac*TPase-mediated remobilization

and by examination of factors known to influence transposition, such as sequence integrity of the 5′- and 3′-TIRs. To study these issues, we developed a population of barley lines, each containing a unique single-copy *Ds* insertion randomly inserted in the genome. Characterization of *Ds* insertions and of their insertion sites, in primary transformants and in progeny populations where transposition was either observed or not, is important for demonstrating the utility of this system as a tool for targeted insertional mutagenesis in barley.

For a targeted gene tagging approach to be successful, it is essential to establish that exogenous *Ds* elements do not lose activity during generation advance due to, for example, methylation of inverted repeat ends or of the transposase gene or because of complications brought about by large numbers of retrotransposons and transposable elements. Some factors we analyze were studied in other crops, particularly dicotyledonous species; however, no such study has been reported in a large genome cereal species. In the current study, data is presented relative to remobilization of *Ds* during generation advance and to the functionality of TNP lines for targeted mutagenesis. Specifically, we present data on integrity of TIR sequences and demonstrate their importance for transpositional competence, and provide qualitative and quantitative characterizations of the ability of *Ds* elements to undergo transposition over four generations, a situation important to the maximal utility of the targeted mutagenesis approach. Molecular and flanking sequence characterization of 50 TNP lines is also reported to validate its marked tendency to transpose into genic regions.

Materials and methods

Development and characterization of TNP lines

Development of the primary TNPs was accomplished by crossing transgenic barley lines expressing *Ac*TPase and barley lines containing a transposition-competent *Ds-bar* element (Koprek et al. 2000). T_2 plants carrying single- or multi-copy *Ds-bar* elements were crossed with plants expressing *Ac*TPase to remobilized *Ds*. Resulting F_1 plants were selfed and F_2 populations analyzed by DNA hybridization for evidence of transposition events, based on differing band migrations. Lines with single, transposed *Ds-bar* copies and no *AcTPase* were made homozygous and designated secondary TNPs. Remobilization of *Ds* elements in five secondary TNPs, i.e., TNP-3, -13, -24, -30, and -41, was accomplished by crossing the lines with an *Ac*TPase-expressing line; F_2 populations obtained from those crosses were screened for new tertiary transposition events. Three tertiary TNPs, JA117-1-106, JA117-1-119, and JA117-1-145, which had *AcTPase*, were advanced to F_3 and quaternary transpositions identified.

Generation and characterization of *Ds* flanking sequences

Plants with single- or low-copy, primary, secondary, tertiary, or quaternary *Ds* transpositions, identified as described above, were analyzed to obtain 5′- and/or 3′-flanking sequences by inverse PCR (iPCR) and TAIL-PCR as described (Cooper et al. 2004). This sequence information was the basis for determining orientation of *Ds* insertions and structure of insertion sites, including TIRs and 8 bp duplications, and to characterize the genomic regions into which transposition occurred.

Generation of overlapping 5′- and 3′-flanking sequences, empty donor sites, and footprints

Separate primers were synthesized for each flanking sequence in reverse orientation (Table 1) to obtain sequences on each side of the *Ds* insertion site in order to match 5′- and 3′-flanking sequences. PCR reactions (25 μl) of genomic DNA contained 1× PCR buffer

Table 1 Primers used for generation of overlapping sequences to match 5′ and 3′ *Ds* flanking regions

Primer	Primer sequence (5′–3′)	Fragment size (bp)
JPT32-5R	CAGACTGCTCCAGATGAGAT	428
JPT32-3R	ACCATCAGCCAGGAGTATGT	
JPT3-5R	AGATAAGGCATTCCGCTTGG	474
JPT3-3R	CCAGCTTGGCAACTTGAACT	
JPT13-5R	CCCTCCCGGTTTGTTTGTGT	332
JPT13-3R	CCAGCTTGGCAACTTGAACT	
JPT18-5R	AATGGAGCACGGACTTGTAG	288
JPT18-3R	GCAGGTAGAGGAAGAAGATG	
JPT19-5R	CCCTGGTCCCAGACTTTACA	210
JPT19-3R	CCGTGCCATGCATAACTAGA	
JPT24-5R	CTGGCTAGCATTAACGTCTC	706
JPT24-3R	GTGGTAGTGCTGGCAGTTCACA	
JPT26-5R	TCGCAACACCACCGCCATTC	487
JPT26-3R	TGGCTAGGCAAGGTGAAGAA	
JPT29-5R	GGTCGACACCTCCACTGTAG	471
JPT29-3R	TACGAACGCACAAGTCACAC	
JPT30-5R	CTTCTCTTCCGACAGCATCA	529
JPT30-3R	CGGCAACATACCATCTTCGG	
JPT81-5R	GCCACCGAAGATGAGTATCC	491
JPT81-3R	TGGCAGTGGTTACTGGCTAC	

(Promega, Madison, WI, USA), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 1 μM primer, 1% DMSO, and 2.5 U Taq DNA polymerase (Promega). PCR reactions were performed with an initial denaturation at 94°C for 2 min, followed by 35 cycles of PCR, with each cycle consisting of 94°C for 30 s, 55°C for 90 s, 72°C for 60 s, and a final 5-min elongation step at 72°C; PCR products were analyzed by gel electrophoresis in 1% agarose gels. For analysis of empty donor sites in primary lines, primers EDS5′ (5′-CGT CAG GGC GCG TCA GCG GGT GTT-3′) and EDS3′ (5′-AAT ACG CAA ACC GCC TCT CCC CGC-3′) were used, as described, to amplify an approximate 300 bp fragment in cases where transposition occurred (Koprek et al. 2000). For analysis of empty donor sites and footprints in secondary lines, primers JPT32B-5R (5′-CAG ACT GCT CCA GAT GAG AT-3′) and JPT32B-3R (5′-ACC ATC AGC CAG GAG TAT GT-3′) were generated from flanking sequences of a primary line, PDS-2, and used to amplify an approximate 350 bp fragment as described above. Fragments from both amplifications were sequenced (Elim Biopharmaceuticals Inc, Hayward, CA, USA). To look for footprints generated in tertiary TNP lines, primers developed from the flanking sequences of a secondary line (TNP-24) from which the tertiary lines originated were used on selected F_2 plants. For tertiary lines, primers RTT24F (5′-ATG TGG TAG TGC TGG CAG TT-3′) and RTT24R (5′-CTG GCT AGC ATT AAC GTC TC-3′) were used to generate an approximate 700 bp fragment, which was subsequently sequenced.

DNA hybridization analysis

To determine copy number of *Ds* insertions, genomic DNA was isolated from young tissues of each TNP. DNA (~15 μg) was digested with either *Eco* RV or *Hind* III, electrophoretically separated on 0.8% agarose gels, transferred to Hybond N^+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA), hybridized with two different ^{32}P-labeled probes (DS 400 and DS 700) and washed according to manufacturer's instructions. The DS 400 probe (400 bp) was generated by digesting *pSP-Ds-Ubi-bar* (McElroy et al. 1997) with *Hind* III and *Eco* RV; the 400 bp fragment contains 340 bp of *Ds* sequence from the 5′-side and 60 bp of the *pSP-Ds-Ubi-bar* backbone. The DS 700 probe was generated by digesting *pSP-Ds-Ubi-bar* with *Kpn* I and *Hpa* I; the 694 bp fragment contains 50 bp of the 3′-end of *bar*, 300 bp of *nos*, 254 bp of *Ds* sequence and 90 bp of the *pSP-Ds-Ubi-bar* backbone. Labeling of the probe was performed according to manufacturer's instructions, using Ready-To-Go™ DNA Labeling Beads (Amersham Bioscience, Buckinghamshire, England, UK).

Bioinformatics analysis

The 5′- and 3′-flanking sequences of TNPs were joined to form a contig using the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Contigs were then queried against databases at TIGR (http://rice.tigr.org/), NCBI (http://www.ncbi.nlm.nih.gov/), Gramene (http://www.gramene.org), and HarvEST (http://harvest.ucr.edu/) using the WU-BLAST algorithm (http://blast.wustl.edu) to search for similarly expressed proteins and ESTs. This analysis also facilitated predictions of exon and intron positions. Genic regions of contigs were also predicted using GenScan (http:// genes.mit.edu). Complete annotations of contigs with regard to *Ds* insertion sites and intron and exon positions were created using Perl and a program (http://nature.berkeley.edu/~cshchen/cgi-bin/take8.pl) utilizing the GD package available on CPAN (http://www.cpan.org/). Six-frame translations of contigs were analyzed using Prosite (http://us.expasy.org/prosite) and the SMART database (http://smart.embl-heidelberg.de/) to search for putative domains.

Results

Development and re-activation of *Ds* insertion lines

A total of 100 barley *Ds* insertion lines were generated in this study, which includes primary, secondary, tertiary, and quaternary transposition events. Re-activation of *Ds* from primary to secondary and secondary to tertiary insertions was achieved by crossing homozygous *Ds* lines devoid of *AcTPase* with *Ac*TPase-expressing lines. Quaternary TNPs were generated after self-pollination of tertiary TNPs, which contained both *Ds* and active *Ac*TPase. The scheme of development and re-activation of TNPs is presented in Fig. 1.

Primary *Ds* insertion lines

A two-rowed barley cultivar, Golden Promise, was co-transformed with a plasmid containing *bar* driven by the maize *ubiquitin1* promoter and first intron, flanked by *Ds* inverted repeat sequences, and with a plasmid containing *AcTPase*, also driven by the *ubiquitin1* promoter and intron (Koprek et al. 2000). Multiple T_0 plants with single- and low-copy number *Ds* elements were generated. The pattern of hybridization, suggesting the copy number of *Ds-bar* in T_2 plants, is

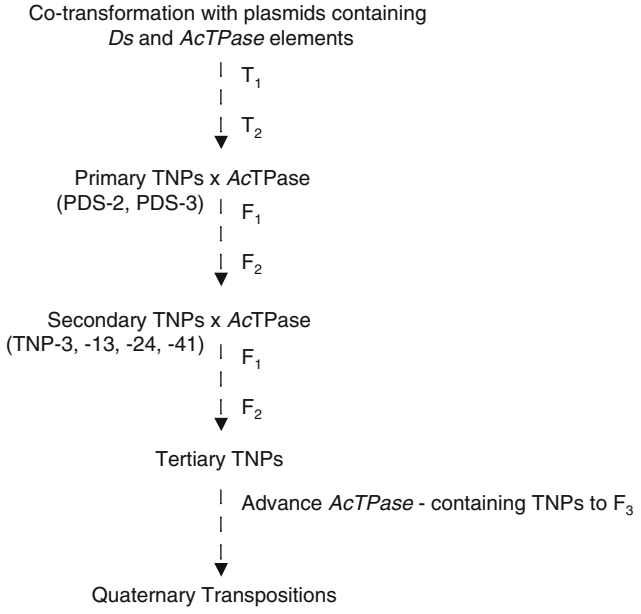

Fig. 1 Summary of *Ds* transposition and reactivation scheme

shown in Fig. 2a. PDS-1 (primary Ds-1) and PDS-2 contain a single copy of *Ds* devoid of *Ac*TPase, whereas PDS-3 contains 3 *Ds* copies including copies of *AcTPase*.

To determine whether the presence of *Ds* elements in the three lines was the result of primary transpositions, rather than to initial transgene integration following bombardment, flanking sequences from T_2 plants were isolated by iPCR and sequenced. None of the *Ds*-flanking sequences matched that from the original plasmid used for transformation (Table 2), indicating these loci were the result of transposition. Although primary transpositions occurred at some time during the generation of T_0 plants, selfing of T_1 plants of PDS-3, containing *AcTPase* and *Ds-bar*, did not yield additional transpositions in the 20 plants analyzed (data not shown); one possibility is that *AcTPase* was inactivated during generation advance of this line.

Both PDS-1 and PDS-2 *Ds* insertions were unique, based on analysis of their flanking sequences. This type of analysis of PDS-3 showed that two of the *Ds* insertions (32B and 32B*x*) corresponded to the insertions in PDS-1 and PDS-2, respectively; a third insertion site (32B*y*) was unique. Thus these loci appeared as independent, unlinked primary transpositions, based on the ability of the 32B and 32B*x* loci to segregate away from each other and from 32B*y* during generation advance. Although exact timing of the primary transpositions is not known, attempts to amplify a 300 bp empty-donor PCR fragment from the three primary lines, using primers specific to sequences flanking *Ds* in the plasmid used to generate the initial transformants, were unsuccessful (data not shown). This indicates that the transpositions may have occurred either as extra-chromosomal activity before *Ds* integrated into the genome or that segregation of these loci from the original site of insertion occurred during regeneration and initial generation advance.

Secondary *Ds* insertion lines

PDS-2 and PDS-3 were chosen for *Ds* remobilization via crossing with *Ac*TPase-expressing plants to generate a population of single-copy TNP lines (Figs. 1, 2b). Both lines had intact TIR sequences (Table 2) and, based on DNA hybridization analyses of progeny derived from these crosses, 43 single-copy *Ds* insertion lines were identified. Crossing the single-copy line, PDS-2, with an *Ac*TPase-expressing plant generated 32 new single-copy TNP lines (TNP-6 to TNP-30, TNP-38 to TNP-41, TNP-51 to TNP-53). Similarly, crossing PDS-3 with an *Ac*TPase-expressing line produced eleven different TNPs (TNP-1 to TNP-5; TNP-31 to TNP-35, TNP-46). Of 43 single-copy lines, a total of 28 secondary TNPs were identified that were devoid of *AcTPase* and contained a single copy of *Ds-bar*—not present at the initial insertion position based on band migration (Fig. 2b).

Tertiary and quaternary *Ds* insertion lines

To induce tertiary transpositons and to investigate the influence of TIR sequence integrity on transposition competence, five single-copy TNP lines, homozygous for secondary *Ds* transpositions, were selected for further study. TNP-3, -24, and -41, which have intact TIRs, and TNP-13 and -30, which have alterations in the TIR sequence, were crossed with *Ac*TPase-expressing lines, and progeny advanced via self-pollination to the F_2 generation (Fig. 1; Tables 3, 4). From each F_2 population, 92–158 individual plants were analyzed by DNA hybridization for evidence of new transpositions. Based on the percentage of plants in which bands with altered migration distances were detected (Fig. 3a), tertiary transposition occurred at a frequency of 13.3% (20 of 150 F_2 plants) for TNP-3; 11.8% (17 of 144) for TNP-41; 17.1% (27 of 158) for TNP-24; 0% for TNP-13 (0 of 99), and 0% for TNP-30 (0 of 92) (Table 3). The TIRs in TNP-13 and TNP-30 are modified at either both the 5′ and 3′ sides or only at the 5′ side, respectively (Table 2).

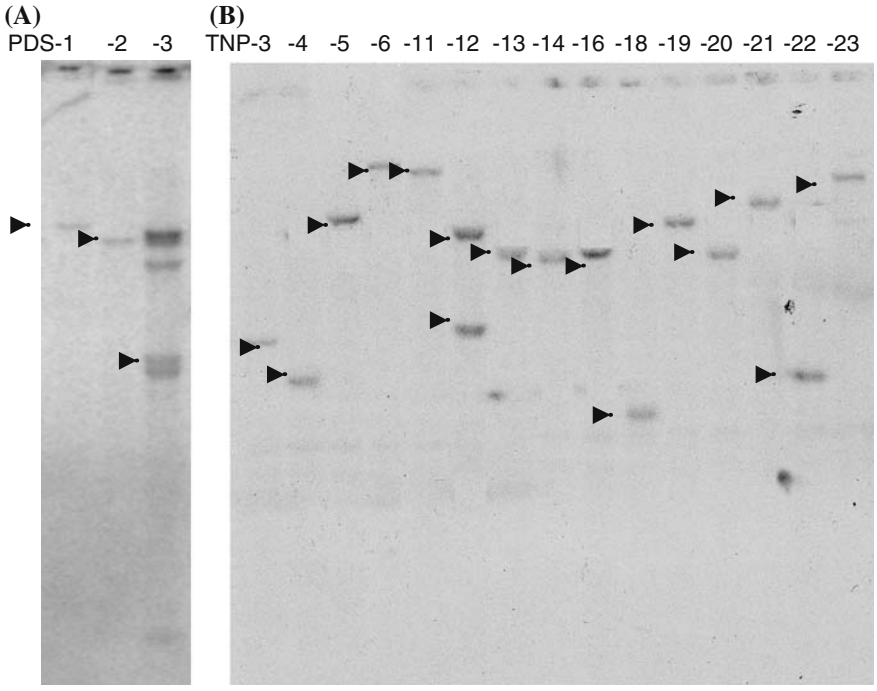

Fig. 2 DNA hybridization analysis of primary and secondary *Ds* transposants. **a** Primary transposition in T_2 generation after co-transformation of *Ds-bar* and *AcTPase* constructs; DNA of PDS-1 (*lane 1*), PDS-2 (*lane 2*), and PDS-3 (*lane 3*), digested with *Hind* III and probed with ^{32}P-labeled fragment containing DS 700. **b** Secondary transpositions observed in progeny of cross of primary transposants, PDS-2 and PDS-3 with *Ac*TPase-expressing plant. TNP-3, -4, -5, -6, -11, -12, -13, -14, -16, -18, -19, -20, -21, -22, and -23 DNA was digested with *Eco* RV and probed with ^{32}P-labeled DS 400; numbers across top indicate particular TNP lines used. *Black arrows* indicate new bands resulting from transpositions

Several lines exhibiting tertiary *Ds* transposition events, in which active *Ac*TPase was present, were selected for additional study. Three F_2 plants from the JA117 population (Table 3) were self-pollinated and a total of 201 F_3 plants were assayed for tertiary transpositions, as described above. Tertiary transposition frequencies were 16.4% for JA117-1-106 (13/79); 13.9% for JA117-1-119 (11/79); and 13.2% for JA117-1-145 (7/53) (Table 3; Fig. 3b).

Generation of plants with multiple transpositions

When plants bearing single-copy *Ds-bar* elements with perfect TIRs were crossed with *Ac*TPase-expressing plants to generate secondary and tertiary lines, some of the resulting plants contained more than one copy of *Ds-bar*. In a population derived from PDS-2 crossed with an *Ac*TPase-expressing line, 14.1% (35/248) of the plants contained *Ds-bar* transpositions (Table 3). DNA hybridization analysis indicated that 40% (14/35) of the lines with transpositions had more than one *Ds-bar* copy and 20% (7/35) had more than two copies. In examining progeny of crosses of the tertiary lines, TNP-3, TNP-24, and TNP-41, with an *Ac*TPase-expressing line, similar observations were made although the frequency of occurrence of multiple copies of *Ds* in two crosses, JA117-1 and JA120-1, was lower than the frequency in secondary transposition populations (Table 4).

Ds excision and footprints

During *Ds* integration an 8-bp duplication of host genome sequence is normally generated at the site of integration (Fedoroff 1989) and, when *Ds* excises, sequence alterations occur in the duplicated copies of genomic sequence; the remaining sequence is referred to as a footprint. The presence of footprints can be observed by analyzing the sequences across empty donor sites, using primers from sequences flanking *Ds* at the integration site; the presence of footprints confirms that both insertion and excision occurred. Using primers from the sequences flanking *Ds* in PDS-2, the predicted, approximate, 350-bp empty donor-site fragment was amplified and sequenced in progeny. Analysis of the alignment of the flanking sequences with the original genomic sequence in the parent indicated that, after the addition of the 8-bp direct repeat, 4–7-base nucleotide deletions occurred in those direct repeats during or after excision (Fig. 4a). In one line, TNP-2, there was a 1-base nucleotide insertion in the original genomic sequence and ‘G’ to ‘C’ transitions.

Excision patterns in a subset of tertiary lines from the cross of TNP-24 and an *Ac*TPase-expressing line were studied using primers from TNP-24 flanking sequences to observe footprints. Patterns of nucleotide deletions in the 8-bp direct repeats of the tertiary lines were similar to those in secondary lines (Fig. 4b), i.e., no insertions and no large deletions were observed.

Table 2 Status of terminal inverted repeats and duplicated sequences at *Ds* insertion sites and annotation of flanking sequences

TNP Line	Status of terminal inverted repeats	Duplicated sequence	Annotation	*e*-value	Database
PDS-1	Perfect (TTTCATCCCTG)	GTCCTAAC	Putative gene	N/A	GenScan
PDS-2	Perfect	GAGCCAAC	Barley EST HA08116u	2e-04	HarvEST
PDS-3	Perfect	CATAACGT	Barley EST BM441822.2	9.9e-08	Gramene
TNP-1	Defective (TTTCATCCTAC)	Not known	Maize EST 3529_1_87_1_D09.y_1	1e-07	TIGR
TNP-3	Perfect	ATTCGTCA	Wall-associated kinase	1.1e-34	TIGR
TNP-6	Perfect	GCTAAATG	Barley EST AV946044.1	1.8e-09	Gramene
TNP-11	Perfect	CACCCAGC	Wall-associated kinase	6.2e-73	TIGR
TNP-13	Defective (TTTCATCCGAT)	AT	Wheat EST wpa1c.pk011.e7	1.5e-07	TIGR
TNP-18	Perfect	TTCCACCG	F-box putative	3.2e-10	TIGR
TNP-19	Perfect	ATGGCACG	Barley EST AJ474918.1	8.8e-19	Gramene
TNP-22	Perfect	CTGAAGAG	Terpene synthase	2.4e-25	TIGR
TNP-24	Perfect	ATTTCATG	RNA binding protein	9.5e-58	TIGR
TNP-25	Perfect	ATTTCATG	Cytochrome P450	6.9e-52	TIGR
TNP-26	Perfect	CTTCCATC	HVSMEc0013O19f	2.4e-35	TIGR
TNP-27	Perfect	AATCGATC	Unknown	N/A	
TNP-28	Perfect	TTTGCGTC	Putative gene	N/A	GenScan
TNP-29	Perfect	TCCCCAGC	Wheat EST wip1c.pk005.a8	4.5e-27	TIGR
TNP-30	Defective (TTTCATCCGCC)	CATGGGC	Expressed protein	9.9e-10	TIGR
TNP-31	Perfect	ACGGCAAT	Putative gene	N/A	GenScan
TNP-32	Perfect	Not known	Phosphate transporter *HvPTv* gene	1e-134	NCBI
TNP-33	Perfect	TCCGCACG	Putative gene	N/A	GenScan
TNP-34	Perfect	ATGTAATG	Unknown	N/A	
TNP-35	Perfect	Not known	Putative gene	N/A	GenScan
TNP-38	Defective (TTTCATCATTG)	Not known	Barley EST HB23D08r	1.2e-18	TIGR
TNP-39	Defective (TTTCATCGACC)	Not known	Wheat EST G608.001G16R011024	4.1e-09	TIGR
TNP-40	Perfect	Not known	Unknown	N/A	
TNP-41	Perfect	CCACGGAG	Ubiquitin conjugating enzyme	2e-54	TIGR
TNP-46	Defective (TTTCATCATTG)	Not known	Wheat EST WHE1148_FO3_LO62S	1e-13	TIGR
TNP-51	Perfect	Not known	Putative gene	N/A	GenScan
TNP-52	Perfect	GATTGAGG	Wheat EST CK151843.1	3.1e-17	Gramene
TNP-53	Perfect	Not known	Wheat EST TAS004.G09R990617	3.3e-11	TIGR
TNP-66	Perfect	Not known	Unknown	N/A	
TNP-67	Perfect	CCATTTGT	ABC transporter	5.1e-148	TIGR
TNP-68	Perfect	Not known	Putative gene	N/A	GenScan
TNP-69	Perfect	Not known	Expressed protein	2.6e-06	TIGR
TNP-71	Perfect	CTTCGAGT	Barley EST CA028167.1	3.5e-35	Gramene
TNP-74	Perfect	TTTTTATT	Putative MLA6 protein	3e-19	NCBI
TNP-79	Perfect	CCCAGGGA	Wheat EST BF200383.1	2.7e-06	Gramene
TNP-80	Perfect	Not known	Pherophorin-like protein	5.4e-19	TIGR
TNP-81	Defective (TTTCATCCGGT)	CATG	Barley EST CX632786.1	1.5e-182	Gramene
TNP-82	Perfect	AGCTTATG	Putative cytochrome P450	2.4e-09	TIGR
TNP-83	Perfect	Not known	ABC transporter	1.4e-81	TIGR
TNP-90	Perfect	GGTTACGC	Barley EST BG310371.1	4.4e-37	Gramene
TNP-92	Perfect	Not known	Barley *Mla* Locus	5e-46	NCBI
TNP-93	Perfect	CGTGTACG	Wheat EST FGAS022629	9e-42	TIGR
TNP-94	Perfect	Not known	Protein kinase domain	8.8e-54	TIGR
TNP-99	Perfect	Not known	Unknown	N/A	
TNP-100	Perfect	Not known	Barley EST HVSMEm0023O17f	2.9e-07	TIGR
TNP-101	Perfect	Not known	Unknown	N/A	
TNP-112	Defective (TTTCATCCCGT)	GCAAA	Unknown	N/A	

Analysis of *Ds* insertion sites

Flanking genomic sequences were determined for 50 primary, secondary, tertiary, and quaternary TNPs and analyzed using public databases and gene prediction programs. For 64% (32/50) of *Ds* insertion sites, it was possible to develop contigs by joining the genomic sequences from the 5′- and 3′-sides of the insertion site (Fig. 5). Analyses of contigs provides information on the structure of the genomic insertion site, as well as the behavior of *Ds* with regard to the integrity of TIRs, 8-bp duplications, tendency of *Ds* to insert into genic regions, the orientation of *Ds* relative to the direction of transcription of the target gene, and the frequencies of particular adjacent nucleotides from the 5′- and 3′-sides. Certain of this information also helped avoid

Table 3 Transposition reactivation frequency of TNP lines from primary to secondary, secondary to tertiary and tertiary to quaternary and status of terminal inverted repeats

Transposition	Cross	Inverted repeat status in parental *Ds* lines	(%) Transpositions
Secondary			
	PDS-2 × 24B-1-4-2 (*Ac*TPase)	Perfect	14.1% (35/248)
	PDS-3 × 25B-1-2-5 (*Ac*TPase)	Perfect	16.9% (19/112)
Tertiary			
	25B-1-1-5 (*Ac*TPase) × TNP-3 (JA 120-1)	Perfect	13.3% (20/150)
	25B-1-1-5 (*Ac*TPase) × TNP-13 (JA 114-1)	Defective	0.0% (0/99)
	TNP-24 × 25B-1-2-3 (*Ac*TPase) (JA 117-1)	Perfect	17.1% (27/158)
	25B-1-2-3 (*Ac*TPase) × TNP-30 (JA 128-1)	Defective	0.0% (0/92)
	25B-1-1-5 (*Ac*TPase) × TNP-41 (JA 112-1)	Perfect	11.8% (17/144)
Quaternary			
	*Ac*TPase-containing JA 117-1-106	Not known	16.4% (13/79)
	*Ac*TPase-containing JA 117-1-119	Not known	13.9% (11/79)
	*Ac*TPase-containing JA 117-1-145	Not known	13.2% (7/53)

Table 4 Numbers of *Ds* transposants in F_2 populations obtained by crossing single-copy *Ds* lines with *Ac*TPase-expressing lines

Cross	(%) Transpositions	Frequency		
		Single copy	Two copies	> Two copies
PDS-2 × 24B-1-4-2 (*Ac*TPase)	14.1% (35/248)	40.0% (14/35)	40.0% (14/35)	20.0% (7/35)
TNP-24 × 25B-1-2-3 (*Ac*TPase) (JA 117-1)	17.1% (27/158)	85.2% (23/27)	11.1% (3/27)	3.7% (1/27)
25B-1-1-5 (*Ac*TPase) × TNP-3 (JA120-1)	13.3% (20/150)	70.0% (14/20)	15.0% (3/20)	15.0% (3/20)
25B-1-1-5 (*Ac*TPase) × TNP-41 (JA112-1)	11.8% (17/144)	47.1% (8/17)	41.2% (7/17)	11.8% (2/17)

possible misinterpretations due to reported presence of inactive, *Ds*-type structures in the barley genome (Chernyshev et al. 1988).

Initially, matching 5′- and 3′-end flanking sequences was undertaken using BLAST searches to identify sequences on both sides of the insertion site that matched the same genic sequence. In some cases, flanking sequence on one side was in an identified genic sequence (EST or BAC), but the sequence from the other side was not—perhaps the latter sequence was from an intron, creating difficulties in establishing that both sequences were part of the same genomic region. This analysis did, however, reveal the presence of 8-bp direct repeats characteristic of *Ds* transpositions. The complementary 8-bp direct repeats then became a means to facilitate the matching of the 5′- and 3′-flanking sequences since the repeat sequences could be found as direct complements on the 5′- and 3′-ends next to the TIRs. Matching of flanking sequences was also confirmed using PCR by amplifying overlapping sequences using primers from 5′- and 3′-flanking sequences (data not shown).

Analyses of flanking sequences from 50 lines indicate that insertion sites of 16% (8/50) of the TNP lines did not contain perfect TIRs and those lines with defective TIRs also had deletions in the direct-repeat genomic sequences. In four TNP lines, TNP-13, TNP-30, TNP-81, and TNP-112, a 2–3 bp deletion in the TIRs and 1–7 bp deletions in the 8-bp footprint sequences were observed.

To determine the nature of *Ds* insertion sites in the barley genome, analyses of *Ds* flanking sequences were conducted, using BLAST searches of public databases, such as those at harvEST, Gramene, TIGR, and NCBI websites, looking for similarities to known proteins, genomic sequences, and/or ESTs. A database of these *Ds*-flanking sequences from barley is available on the GrainGenes website (http://wheat.pw.usda.gov/BarleyTNP); characterized flanking sequences are available on GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). Annotations of these searches are summarized in Table 2. Based on BLAST searches, 72% (36/50) of TNP flanking sequences match known ESTs or characterized gene sequences (Table 2). Analyzing these flanking sequences with the gene prediction program, 86% (43/50) of transpositions are believed to be in genic regions (Fig. 5).

Ds was found in an exon in the primary transposition line, PDS-2, and within 300 bp of gene-coding regions in PDS-2 and PDS-3 (Fig. 5). Similar trends were observed in secondary and tertiary lines, where 89% (25/28) and 79% (15/19) of insertions, respectively, were in verifiable genic regions (Table 2). Detailed analyses of contig sequences, using searches

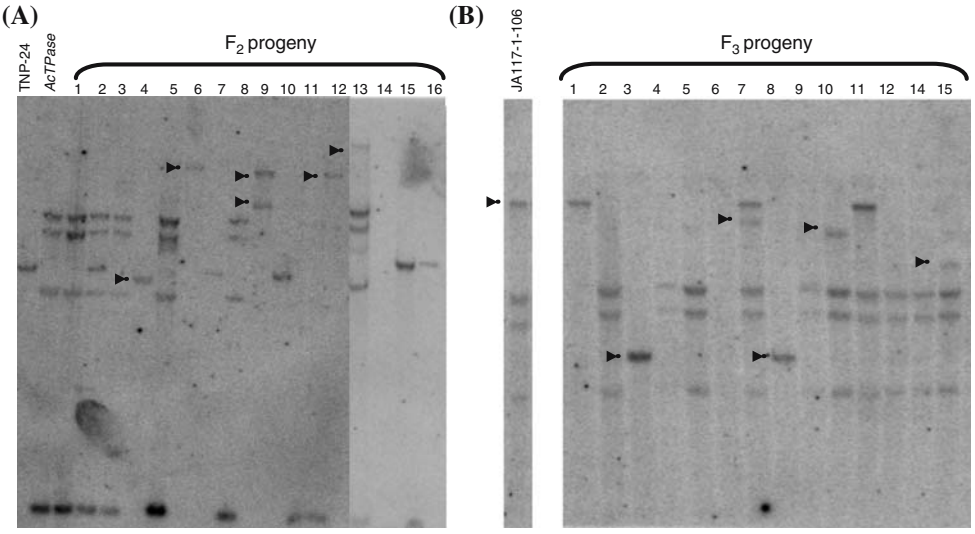

Fig. 3 Analysis of tertiary and quaternary *Ds* transpositions from F_2 population obtained from crosses of TNP-24 and *Ac*TPase lines. **a** Plants with tertiary transpositions: TNP-24 (*lane 1*), *Ac*TPase line (*lane 2*), F_2 population derived from TNP-24 and *Ac*TPase cross (*lanes 1–16*). DNA digested with *Hin*dIII and probed with ^{32}P-labeled Ds700. *Black arrows* indicates transpositions. **b** Plants with quaternary transpositions. JA117-1-106 (*lane 1*); F_3 generation from F_2 plant JA117-1-106 containing tertiary *Ds* transposition and *Ac*TPase (*lanes 1–15*). DNA digested with *Hin*dIII and probed with ^{32}P labeled DS 700; *black arrows* indicate transpositions

of available databases, revealed intron and exon regions, putative domains and translational initiation and termination codons in the *Ds*-flanking genic regions (Fig. 5). Flanking sequences of 17 TNPs contained either predicted ATG initiation codons or stop codons; flanking sequence from one line, TNP-39, contained both start- and stop-codons.

The predicted orientation of transcription of the gene into which *Ds* transposed was compared with the orientation of the *Ds* TIRs. *Ds* insertion orientation was in the same orientation as the gene in 60.4% (26/43) of the lines; in 39.5% (17/43) of the lines it was not. Frequencies of individual nucleotides adjacent to the TIRs were determined in 42 lines, after excluding eight lines with defective TIRs; the adjacent nucleotide was G or C in 61.9 % (26/42) of the cases, while the second and third nucleotides were A or T in 64.2 and 52.3% of the cases, respectively.

Discussion

Transposon-based gene tagging approaches are being developed in *Arabidopsis* and rice, aided by the

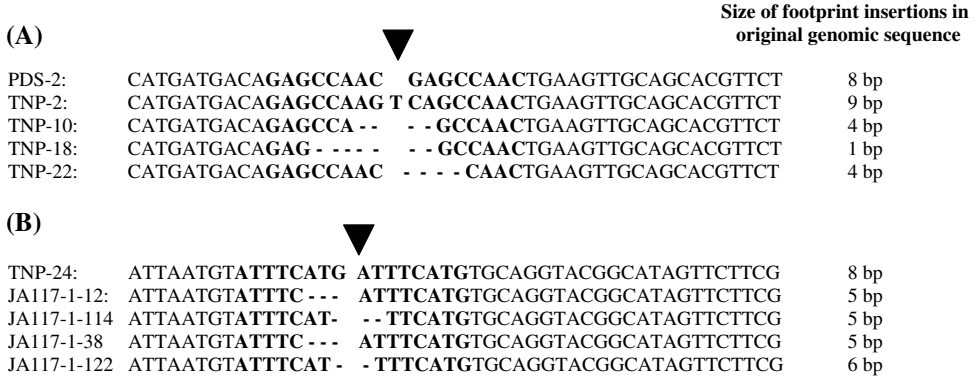

Fig. 4 Analysis of empty donor sites following activation of primary and secondary *Ds* transposants. **a** PDS-2, a primary transposant, is the original line for other secondary TNP lines listed. **b** TNP-24, a secondary TNP line, is the original line for other tertiary TNP lines listed. Direct repeats in primary and secondary TNP lines are shown in *bold*. Numbers to left of sequences indicate the TNP lines screened for excision. Numbers to right of sequences indicate number of bp in footprint insertions observed in TNP lines compared to wild type genomic sequences. *Black arrows* indicate insertion site of *Ds*

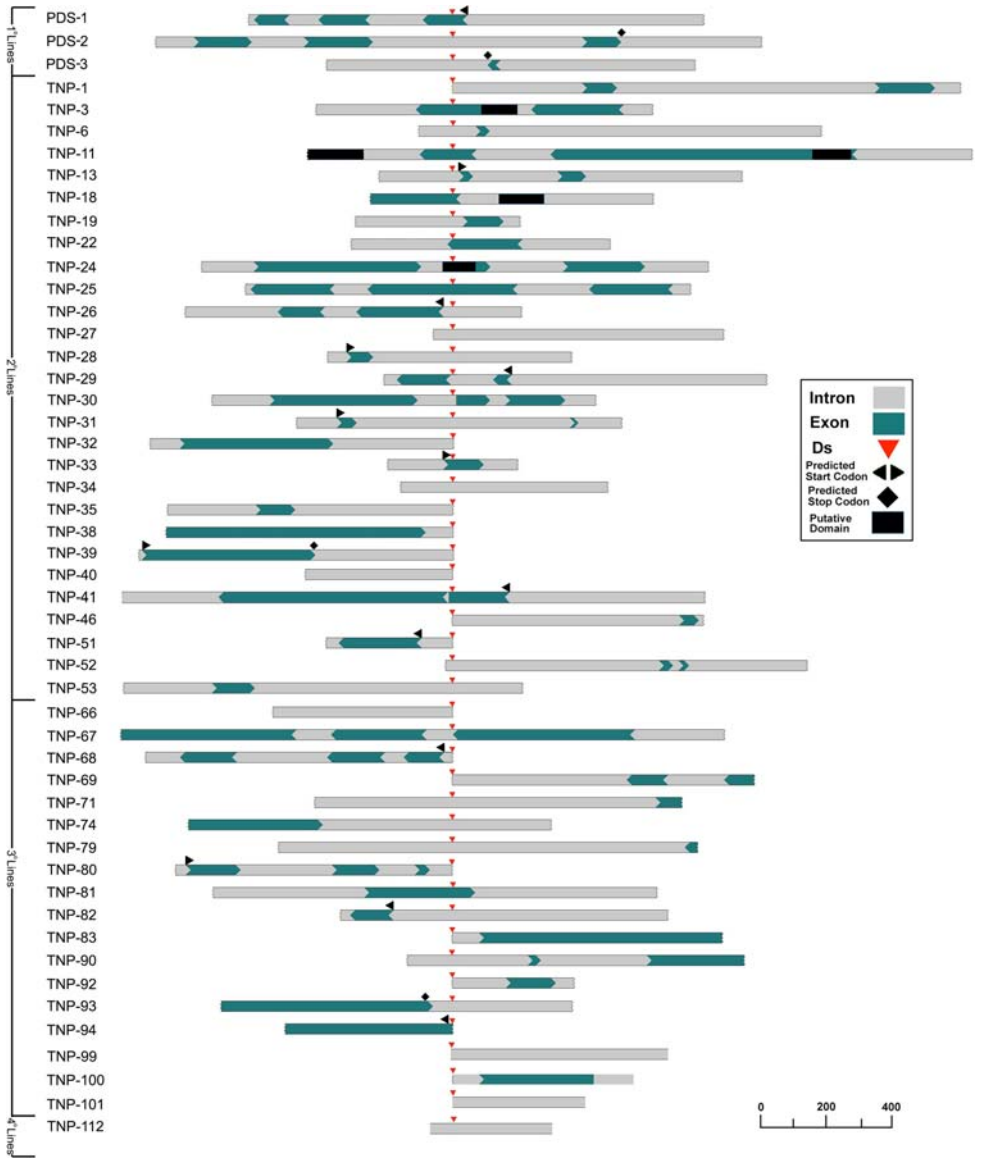

Fig. 5 Flanking sequence contigs from primary, secondary, tertiary, and quaternary *Ds* insertions. Exon, intron, *Ds*, putative start and stop codons, and domain positions are indicated

availability of their complete genome sequences and ease of transformation (Kolesnik et al. 2004; Kuromori et al. 2004). In larger genome species, like barley and wheat, use of transposons for such functional studies was delayed, perhaps due in part to the extensive synteny with rice that was thought by some to preclude the need for such a tool in these cereal species. However, comparative studies have revealed extensive variation in the different cereal genomes (e.g., Bennetzen and Ma 2003; Caldwell et al. 2004). For example, comparison of the rice genome with that of wheat (*Triticum aestivum* L.), barley, maize, and sorghum (*Sorghum bicolor* L.) demonstrates that rapid evolution resulted in significant DNA sequence variation (e.g., Keller and Feuillet 2000) among monocotyledonous *Triticeae* species. As barley is a true diploid and shares a high degree of synteny with wheat, developing an efficient tagging system in barley will be very valuable for both species.

High-frequency remobilization of *Ds* during multigenerational studies

To ensure maximal utilization of mapped TNPs for localized gene tagging, it is imperative to understand factors that are important for *Ds* remobilization during generation advance. The frequency of secondary transposition in our study was 14.1–16.9%, after correcting for TNP redundancy. Koprek et al. (2000) reported higher frequencies of secondary transpositions (up to 47%) in barley; however, flanking sequence analysis was not performed, and therefore

some lines scored as unique may have been redundant. Consistent with the results in the present study, Upadhayaya et al. (2002) noted an average *Ds* transposition frequency of 6.6–11.5% in rice, the only other heterologous cereal in which this transposon system has been studied in detail. Kolesnik et al. (2004), in contrast, reported a higher *Ds* transposition frequency (51%) in rice, perhaps because of redundancies or to differences in promoter constructs. *Ac*TPase was driven by CaMV 35S promoter in the Kolesnik et al. study (2004) and by maize *ubiquitin1* in our study and those of Upadhayaya et al. (2002). This difference in promoters might have caused alterations in timing and hence frequency of *Ds* transposition, as was observed in *Arabidopsis* (Bancroft and Dean 1993; Balcells and Coupland 1994; Smith et al. 1996).

In certain studies of *Ds* reactivation in rice, transposition frequencies declined in later generations. For example, *Ac*TPase-containing F_2 *Ds* plants, transformed by electroporation were advanced to F_4 (Izawa et al. 1997) and *AcTPase*-containing *Ds* plants, created by *Agrobacterium* infection, were observed up to third round of tillering (Chin et al. 1999). In both cases, transposition was rarely seen in later generations (F_3, F_4, and third tillering), although only small numbers of plants were observed. Chin et al. offered no explanation for the decline; Izawa et al. speculated that the decrease was due to *Ds* inactivation. Greco et al. (2003) also observed a decline in *Ds* transposition efficiency during generation advance of rice, attributing it to the methylation status of *Ds*. Activation of single-component *Ac* insertions in rice underwent a pronounced reduction in frequency of transposition as the level of genomic cytosine methylation increased in dedifferentiated cell cultures (Kohli et al. 2004).

In very recent studies by Szeverenyi et al. (2006) in rice *Ds* appeared to retain its activity for several generations. This observation is consistent with results in our study where we observed no decline in transposition frequency in later generations, except for elements (i.e., TNP-13 and TNP-30) with defective TIRs. Although expression from *bar*, inserted between *Ds* inverted repeat sequences, was stable in barley in single-copy, *Ds-bar* insertion lines over six generations (Koprek et al. 2001; Meng et al. 2006), this does not relate directly to the methylation status of the *Ds* ends flanking *bar*. The fact remains, however, that we did not observe decreased transposition frequency following generation advance, so either *Ds* ends were not methylated or methylation of the element does not adversely affect its ability to transpose.

Transposition frequencies of 13.2–16.4%, similar to those observed in secondary populations, were observed in quaternary populations generated when newly transposed *Ds* elements were generation-advanced in the presence of *AcTPase*. The ability to activate and obtain quaternary transpositions in the manner described strongly favors the targeted mutagenesis approach that will be critical in barley and wheat. For example, this allows for the repeated remobilization needed for ''transposon walking'', i.e., sequential localized transpositions, that can be used to tag members of clustered gene families.

Transposition ability correlates with intact TIRs

Studies in maize indicate there are motifs in the subterminal sequences of *Ac* involved in its ability to transpose (Kunze and Starlinger 1989). In fact, it was shown that deletions in the 11 bp TIRs make *Ac* immobile in tobacco (Hehl and Baker 1989) and greatly impair its transposition in other species (Chatterjee and Starlinger 1995; Xiao and Peterson 2002), although *Ac*TPase expression can still result in transposition of other *Ds* elements. In the present study, we also investigated the relationship between imperfect TIRs and the ability of the resulting *Ds* to transpose. For this purpose, three TNPs with perfect TIRs (TNP-3, -24, and -41) and two TNPs with altered TIRs (TNP-13 and -30) were crossed with *Ac*TPase-expressing lines. DNA hybridization analysis from F_2 populations indicates that remobilization was relatively high in lines in which TIR sequences are intact; however, it is completely abolished in lines where TIR sequences are modified. Of 50 TNP lines studied, 16% have imperfect TIRs, suggesting that aberrant insertion/excision of *Ds* occurs frequently and should be considered in selecting candidate lines to reactivate for targeted mutagenesis.

The state of the small 8-bp, direct-repeat footprints, juxtaposed immediately next to the TIRs, was also investigated. Analysis of our results reveals a strong correlation between perfect TIRs and complete 8-bp duplications and the converse—between defective TIRs and imperfect 8-bp direct repeat footprints. In all lines where TIRs are altered (16%; 8/50), deletion of 1–7 bp was also observed in the 8 bp direct repeats. In *Arabidopsis*, a higher frequency (56%) of TNP lines with both defective TIRs and defective 8 bp duplications was observed (Ito et al. 2002).

We observed small 1–9-bp insertion footprints, consistent with observations in maize and *Arabidopsis* where the addition of a few bases at the site of *Ds* insertion were observed (Bancroft et al. 1993; Scott et al. 1996). A deletion of 52 bp was observed at the site of insertion in one barley study (Scholz et al. 2001), but we observed no large deletions in footprints. Since

deletions and insertions of genomic sequences occur in footprints following transposition, generation of such mutations in these genic regions opens new avenues to utilize transposons to generate new alleles and alter the function and expression of target genes.

TNP insertion occur in genic regions

A high frequency of *Ds* insertions into exons or close to exons is important for gene isolation and reverse genetics studies in barley and wheat, where a high percentage of the genome is composed of repetitive regions and transposable elements (Shirasu et al. 2000; Wicker et al. 2005). Using bioinformatic tools to analyze flanking sequences from the 5′- and 3′-ends of 50 TNPs, we studied this issue in detail, demonstrating high-frequency *Ds* insertion either into genes or in close proximity to genes. BLAST searches of databases in TIGR, NCBI, and HarvEST reveal that 72% of TNP flanking sequences matched ESTs or characterized gene sequences; analysis with a gene prediction program identified 86% of *Ds* insertions as being in genic regions. Similar observations were obtained in maize, where 75% of *Ac* insertions were in genic regions (Cowperthwaite et al. 2002), and in rice, where 72% of *Ds* transpositions were in genic regions (Kolesnik et al. 2004). In *Arabidopsis*, *Ds* was found to transpose preferentially into the 5′-ends of genes (Parinov et al. 1999), although in rice *Ds* was found to insert randomly with respect to exons and introns (Cowperthwaite et al. 2002; Kolesnik et al. 2004). In barley, we observe a high frequency (70%; 35/50) of *Ds* transpositions in introns/intergenic regions. Although the implication of this observation on gene expression needs further investigation, the tendency of *Ds* to transpose into genic regions remains an extremely useful tool for gene identification in large genome cereals.

Use of *Ds* for functional genomics and tagging of closely linked genes

Interrupting a specific gene and generating a loss of function phenotype is not straightforward in any plant species because the tissue, circumstance, and developmental stage in which an unknown gene is expressed must be determined. Large-genome species have the additional challenges of low-gene density and gene redundancy. Numerous putative genes were tagged in our study including wall-associated kinases, ABC transporter protein, ubiquitin conjugating enzyme, terpene synthase, F-Box protein, MLA loci, and cytochrome P450. Development of knock-out lines in these genes and in other tagged, ESTs of unknown function provide a useful starting point from which to investigate function by observing expression profiles under a variety of conditions using, for example, RT-PCR and microarray analyses. Toward this end, preliminary RT-PCR analyses, using primers from predicted exons of 12 TNPs, revealed that in 58% (7/12) of the cases expression of the interrupted gene was disrupted in RNA samples from specific developmental stages and under certain environmental conditions (data not shown).

Developing large numbers of mapped TNPs provides the opportunity for saturation mutagenesis of linked genes, made possible because of the ability to remobilize TNPs and their marked tendency to transpose to closely linked locations. In *Arabidopsis* approximately 50% of the time *Ds* transposes to sites within 30 cM of the donor site (Smith et al. 1996; Parinov et al. 1999; Raina et al. 2002). Transposition to linked sites in rice was estimated to be high: 62–67%, based on marker gene segregation (Upadhyaya et al. 2002), and 80–100%, based on empty-donor site analysis (Nakagawa et al. 2000). In barley preliminary estimates were in the range of 75%, based on segregation analysis of *Ds* and *AcTPase* (Koprek et al. 2000).

Data in the present study on multigenerational *Ds* re-activation is critical for localized saturation mutagenesis efforts, including the re-activation needed for ‘‘transposon walking’’, the sequential re-activation of *Ds* that can be used to identify members of clustered gene families. Since no published data is available in barley or in its close relative, wheat, that addresses the capability for consecutive *Ds* remobilization during generation advance, analysis of the four-generation advancement described in this study establishes that the frequencies of re-activation are more than adequate to conduct saturation mutagenesis in barley and wheat of linked regions, setting the stage for reactivation tagging of loci proximal to mapped TNPs.

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